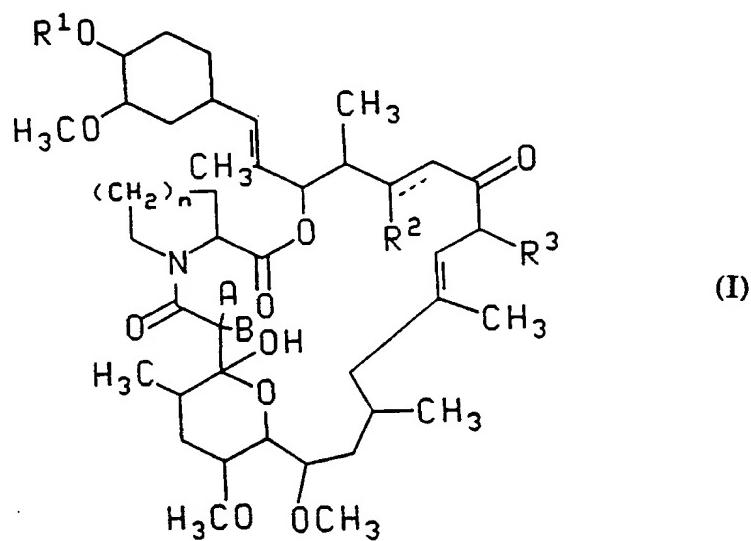




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(54) Title: 2-AMINOSUGAR DERIVATIVES OF MACROLIDES



(57) Abstract

Macrolides of formula (I) and methods of treatment of resistance to transplantation, fungal infections and autoimmune diseases such as rheumatoid arthritis and psoriasis using said macrolides of formula (I), wherein n is 1 or 2; A and B are taken together and form =O or A and B are taken separately and are each H or A is OH and B is H; R¹ is a 2-aminoglycosyl group; R² is OH or a glycosyloxy group; and R³ is an alkyl or allyl group.

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2-AMINOSUGAR DERIVATIVES OF MACROLIDES

Background of the Invention

This invention relates to new chemical compounds which have value in the field of medical science. More particularly, it relates to new chemical compounds which are of value for administration to a mammalian subject, particularly man, as immunosuppressive agents. These new immunosuppressive agents can be compared to the macrolides known as FK-506 and FK-520, which are described in further detail in United States Patent No. 4,894,366. The new compounds of this invention will find special utility in preventing or treating graft rejection following skin or organ transplant surgery and in preventing or treating autoimmune diseases such as rheumatoid arthritis and psoriasis. Additionally, these macrolide derivatives will find use in preventing or treating infectious diseases caused by fungi.

Graft or organ transplant rejection following transplant surgery is a common occurrence which arises when foreign antigens are recognized by the host's immune response system. The host's immune response system, in an effort to "protect" itself from the foreign tissue, then releases its cellular and humoral arsenal. The antibodies attack the foreign tissue, resulting in complications which often end in rejection of said tissue.

Similarly, the occurrence of immunoregulatory irregularities in autoimmune and chronic inflammatory diseases is well known. Irrespective of the underlying etiology of the condition, a variety of autoantibodies and self-reactive lymphocytes often arise to complicate the condition.

Treatments which target the immune response system often result in a complete shutdown of the system, leading to a lowering of the body's ability to combat infection. This can be as dangerous as the original condition which led to the shutdown.

Currently the leading medicinal agent for the prevention or treatment of graft rejection is cyclosporin A, approved by the United States Food and Drug Administration in 1983. The drug acts by inhibiting the body's immune response system from mobilizing its arsenal of natural protecting agents to reject the transplant's foreign protein. Although cyclosporin is effective in fighting graft rejection, it suffers drawbacks in that it can cause kidney failure, liver damage and ulcers; which in many cases can be very severe. Safer drugs which are more selective in their ability to affect the

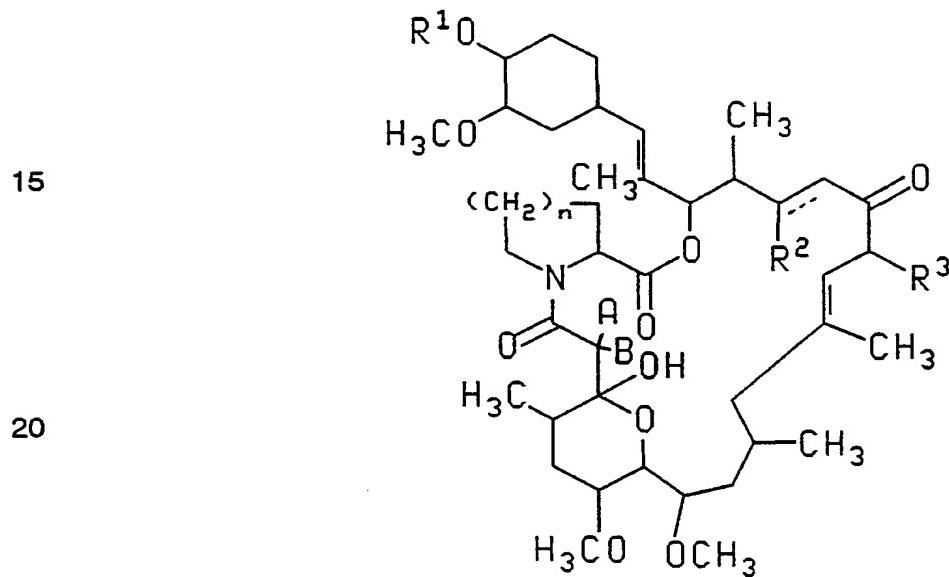
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immune response system and which have fewer side effects are constantly being pursued.

United States Patent No. 4,894,366 discloses the macrolides FK-506 and FK-520, *inter alia*, as immunosuppressants, including the treatment of "resistance to transplantation," autoimmune diseases and infectious diseases. International Patent Publication No. WO 91/02736 discloses derivatives of FK-506, FK-520 and related macrolides. European Patent Publication No. 428,365 A1 discloses various other derivatives of FK-506, FK-520 and related macrolides.

Summary of the Invention

10 The present invention is directed to compounds of the formula



25

(I)

or a pharmaceutically acceptable salt thereof;

wherein n is 1 or 2;

the dotted line represents an optional double bond in the case where R² is H;

30 A and B are taken separately and A is H and B is H or OH, or A and B are taken together and form =O;

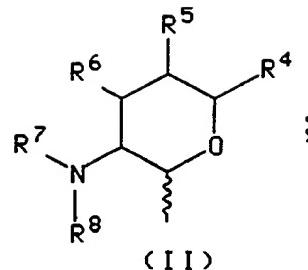
R² is H, (C₂-C₅)alkanoyloxy or -OR⁰;

R³ is (C₁-C₃)alkyl or allyl;

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R¹ and R⁹ are each H or

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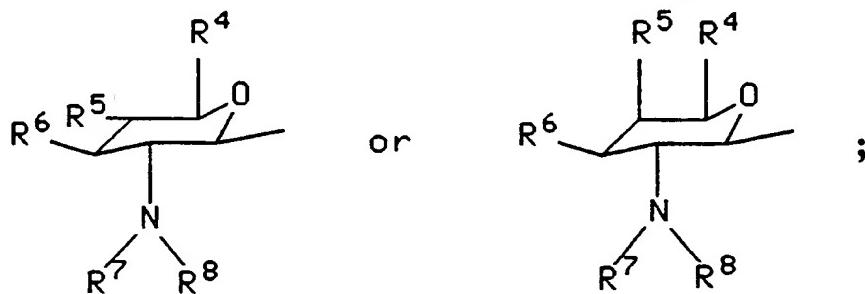


- R⁴ is, for each occurrence, independently -CO₂R⁹, -CO₂H, -CH₂OH, H, -CH₃, -CONH₂, -CONHR⁹, -CONR₂⁹, -CH₂OCOR⁹, -CH₂OCO₂R⁹, -CH₂OCONHR⁹, -CH₂OCONR₂⁹ or -CH₂OR⁹;
- 10 R⁵ and R⁶ are, for each occurrence, independently (C₁ to C₄)alkoxy, benzyloxy, -OH, -OCOR⁹, -OCO₂R⁹ or -OSiR₃¹⁰;
- 15 R⁷ and R⁸ are, for each occurrence, independently H, (C₁ to C₄)alkyl, -COCH₂R⁹, -COR⁹, -CO₂R⁹ or -SO₂R⁹;
- R⁹ is, for each occurrence, independently (C₁ to C₆)alkyl, (C₃ to C₆)cycloalkyl, allyl, -CF₃, pyridyl, thienyl, thienylmethylene, furanyl, benzyl, benzyl variously substituted with one to five halogen atoms, -OH groups or (C₁ to C₄)alkoxy groups, phenyl or phenyl substituted with one to five halogen atoms, -OH groups or (C₁ to C₄)alkoxy groups; and
- 20 R¹⁰ is, for each occurrence, independently (C₁ to C₄)alkyl, phenyl or benzyl; provided that R¹ and R⁹ are not both H.

A preferred group of compounds of this invention is the group of compounds of formula (I) wherein n is 2; the dotted line represents no bond, A and B are taken together and form =O; R² is -OH; R³ is ethyl; R¹ is

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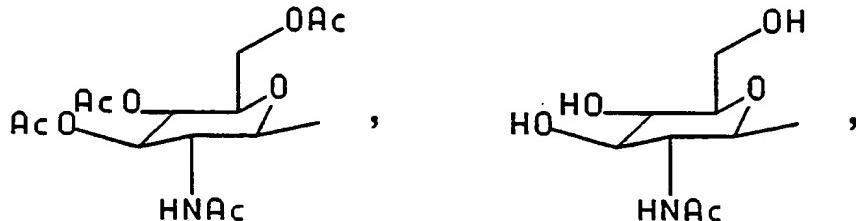


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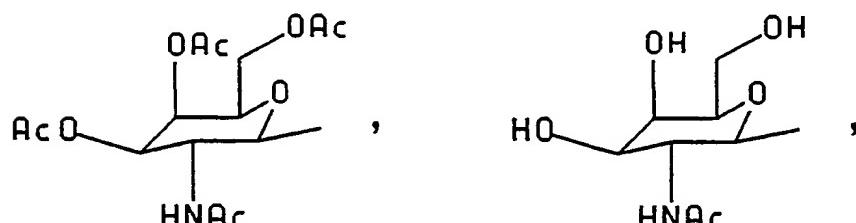
R^4 is $-\text{CH}_2\text{OH}$ or $-\text{CH}_2\text{OCOCH}_3$; R^5 and R^6 are each independently $-\text{OH}$ or $-\text{OCOCH}_3$; and R^7 and R^8 are each independently H , $-\text{COCH}_3$ or $-\text{COCF}_3$.

Especially preferred within this group are the compounds where R^1 is

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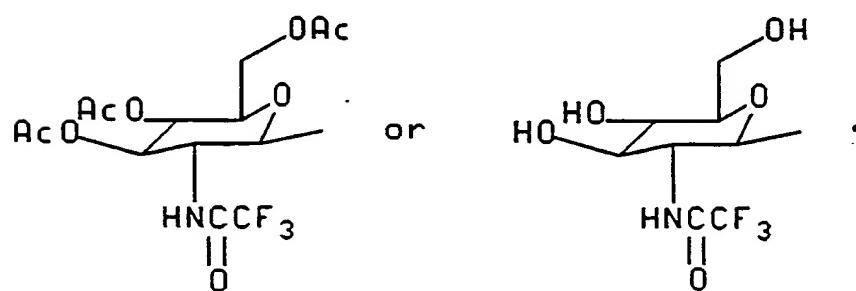


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A second preferred group of compounds of this invention is the group of compounds of formula (I) wherein n is 2; A and B are taken separately and are each H; the dotted line represents no bond; R² is -OH; and R³ is ethyl.

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The compounds of formula (I) are active as immunosuppressants. This activity makes these compounds useful in treating and preventing graft and transplant rejection. Further, this activity makes these compounds useful in preventing and

treating autoimmune diseases such as rheumatoid arthritis and psoriasis in a mammal, especially man.

Accordingly this invention also embraces a method of treating resistance to transplantation in a mammal in need of such treatment comprising administering to said 5 mammal a compound of formula (I) or a pharmaceutically acceptable salt thereof.

The term "transplantation," when used above and hereinafter, refers to the implantation in one part of an individual of a tissue or organ taken from another part of that individual or from another individual. Typical transplants include, but are not limited to, bone marrow, heart, renal, tendon and pancreaticoduodenal 10 transplants.

The term "graft" when used above and hereinafter, refers to any unattached tissue or organ which is used for transplants. Typical grafts include, but are not limited to, skin, bone, fat and nerve grafts.

Additionally this invention embraces a method of treating autoimmune disease 15 (such as rheumatoid arthritis or psoriasis) in a mammal in need of such treatment comprising administering to said mammal an effective amount of a compound of formula (I) or a pharmaceutically acceptable salt thereof.

Further, this invention embraces a pharmaceutical composition comprising a resistance to transplantation treating effective amount of a compound of formula (I) and 20 a pharmaceutically acceptable carrier.

Still further this invention embraces a pharmaceutical composition comprising an autoimmune disease (such as rheumatoid arthritis or psoriasis) treating effective amount of a compound of formula (I) and a pharmaceutically acceptable carrier.

Yet further the compounds of this invention of formula (I) have antifungal activity. 25 Hence these compounds can be used to treat or prevent infections in mammals caused by fungi.

Accordingly, this invention embraces a method of treating diseases caused by fungi in a mammal in need of such treatment comprising administering to said mammal an effective amount of a compound of formula (I) or a pharmaceutically acceptable salt 30 thereof.

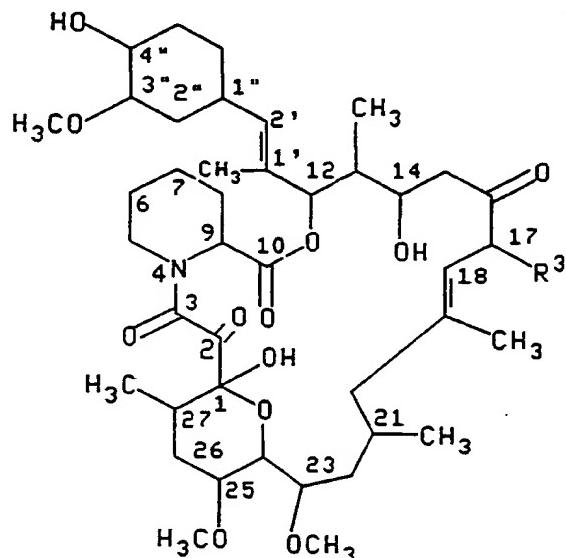
Additionally, this invention embraces a pharmaceutical composition comprising a fungal infectious disease treating effective amount of a compound of formula (I) and a pharmaceutically acceptable carrier.

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Detailed Description

The compounds of formula (I) of the present invention are readily prepared. Most generally, a macrolide of formula (III) or (IV) below

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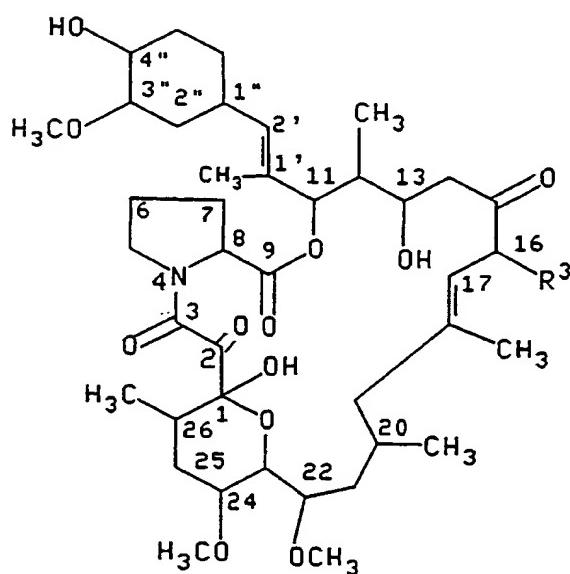


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(III)

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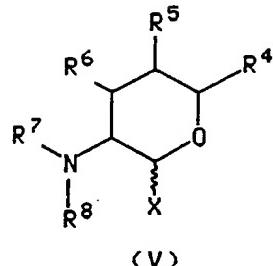
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(IV)

is coupled with an appropriate sugar halide derivative of the formula

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wherein X is halo, such as bromo or chloro. The coupled (or glycosylated) macrolide
10 is then further modified as described hereinbelow.

The production of macrolides of formulae (III) and (IV) is well-known in the literature. The generally preferred route to these macrolides is *via* biological fermentation of microorganisms belonging to the genus *Streptomyces*. The compounds of formulae (III) and (IV) wherein R³ is allyl are obtained by fermentation
15 of *Streptomyces tsukubaensis* No. 9993 (Ferm BP-927). The compound of formula (III) wherein R³ is ethyl and the compound of formula (III) wherein R³ is methyl are obtained by fermentation of *Streptomyces hygroscopicus* subsp. *ascomyceticus*
ATCC 14891.

A lyophilized sample of *Streptomyces hygroscopicus* subsp. *ascomyceticus*
20 ATCC 14891 has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852, U.S.A., under the terms of the Budapest Treaty on January 13, 1992. This newly deposited culture was given the new deposit number of ATCC 55276.

Streptomyces tsukubaensis No. 9993 (Ferm BP-927) is currently on deposit with
25 the Fermentation Research Institute, Agency of Industrial Science and Technology (No. 1-3, Higashi-1-chome, Yatabemachi, Tsukuba-gun, Ibaraki Prefecture, Japan), under the provisions of the Budapest Treaty. A fresh sample of the microorganism will be deposited with the American Type Culture Collection in accordance with the terms of the Budapest Treaty.

30 The above-mentioned microorganisms, when placed separately in aqueous nutrient media, will produce the aforementioned compounds of formulae III and IV. The fermentation of said microorganisms to produce these macrolides is accomplished substantially as disclosed in U.S. Patent 4,894,366, which is hereby incorporated by

reference. Any changes made to the disclosed procedure are made in order to accommodate existing equipment at the facility and are described in Preparations 1 and 2 hereinbelow.

To prepare the compound of formula (I) wherein R⁰ is H and R¹ is a sugar 5 substituent of formula (II), a macrolide of formula (III) or (IV) is coupled with a sugar halide of formula (V). The coupling (or glycosylation) reaction of a sugar halide of formula (V) and a macrolide of formula (III) or (IV) is accomplished in a straightforward manner, using chemistry well known to one of ordinary skill in the art. The coupling reaction is generally carried out using the peracetylated form of the sugar. About 2-4 10 molar equivalents of the appropriate sugar halide of formula (V) is mixed with the macrolide of formula (III) or (IV) in a reaction inert solvent. Reaction inert solvents useful for this type of reaction include chlorinated solvents such as chloroform, methylene chloride and ethylene dichloride; ether solvents such as diethyl ether, tetrahydrofuran, dioxane and dimethoxyethane; aromatic solvents such as benzene, 15 toluene and xylene; and dipolar aprotic solvents such as N,N-dimethylformamide, acetonitrile and N-methylpyrrolidone. Preferred solvents are chlorinated solvents and a particularly preferred solvent is methylene chloride. Generally it is desirable to employ enough solvent such that the reactants are dissolved or suspended by the solvent. Typically the amount of solvent used is varied to give a 10⁻¹ to 10⁻³ Molar solution of 20 macrolide with 10⁻¹ Molar being preferred. Dry conditions are maintained during the course of the reaction by the utilization of anhydrous solvents and by the addition of a drying agent to the reaction mixture. Drying agents typically used for this purpose are molecular sieves, calcium sulfate and magnesium sulfate. A preferred drying agent is 4Å molecular sieves.

25 Initial mixing of the reagents is performed at a temperature of from about -78°C to about 70°C. Preferred are temperatures ranging from about -78°C to about 0°C. Especially preferred for ease of preparation is a cooling bath which maintains the reaction temperature at -78°C.

After the above-mentioned reactants have been mixed and the temperature has 30 equilibrated to -78°C, the reaction mixture is treated with a suitable base such as mercuric carbonate, silver carbonate, mercuric nitrate or silver nitrate. The preferred base for this reaction is silver carbonate. Following addition of said base, the reaction mixture is treated with a catalyst. Typical catalysts for this reaction include triflate,

perchlorate and tetrafluoroborate salts of the cation associated with the particular base used. The preferred catalyst is silver triflate.

After all reactants and reagents have been added, the reaction mixture is warmed to 0°C, stirred for 0.5-24 hours at 0°C and then warmed slowly to room temperature. The reaction mixture is stirred for an additional 0.5-24 hours at room temperature. Generally, the reaction mixture is stirred at 0°C for 5 hours and allowed to warm to room temperature over 3 hours followed by stirring at room temperature for 16 hours. The product is then isolated from the reaction mixture using techniques familiar to one of ordinary skill in the art. Thus, simple filtration through a filter aid such as Celite® followed by evaporation affords a residue which is purified by column chromatography. One of ordinary skill in the art will recognize that column chromatography entails the use of a solid phase component such as silica gel and a liquid phase component comprised of an advantageous mixture of solvents for the separation and purification of compounds from a mixture. Removal of solvents after chromatography affords the glycosylmacrolide. Generally, the coupling reaction only takes place at one of the three alcohol sites of the macrolide, this site being the C-4" alcoholic functionality (see Formula III). This selectivity is possibly due to the greater availability of the hydroxyl group of this position in the macrolide's preferred conformation. On occasion, however, with particularly reactive sugar halides, small amounts of diglycosylated material (wherein R² = OR⁰) are formed. This material is detected during the monitoring of the progress of the reaction, which is generally accomplished *via* thin layer chromatography, according to standard practice. The diglycosylated material is isolated and purified as for the monoglycosylated material with the notable exception that the diglycosylated material is generally the first material isolated from the chromatography, with the monoglycosylated material being isolated in later fractions. The use of added equivalents of sugar chloride or the altering of other parameters such as solvent, base or catalyst can affect the yield of diglycosylated material.

To prepare the compound of formula (I) wherein R¹ is H and R⁰ is a sugar substituent of the formula (II), a macrolide of formula (III) or (IV) is first protected with a hydroxyl protecting group at the C-4" position. Hydroxyl protecting groups suitable for such purposes include but are not limited to such groups as silyl ethers, carboxylic esters and carbonic esters of the alcohol. The protecting groups are appended to the

-10-

alcohol utilizing the well known methods of organic chemistry. Bulky silyl ethers are preferred for their selectivity, ease of attachment and ease of removal. Conveniently, a macrolide of formula (III) or (IV) is dissolved in a reaction inert solvent at a temperature of about 0°C to about 30°C. Reaction inert solvents for this type of reaction 5 include dipolar aprotic solvents such as dimethylformamide, dimethylsulfoxide and N-methyl pyrrolidone; chlorinated solvents such as chloroform, dichloromethane and 1,2-dichloroethane; and ether solvents such as diethyl ether, dioxane and tetrahydrofuran. The solvent of choice is often dimethylformamide. A silylating agent, usually a silyl trifluoromethanesulfonate such as t-butyldimethylsilyltrifluoromethanesulfonate or a 10 silyl chloride such as dimethyl-t-butylsilyl chloride, trimethylchlorosilane or triphenylchlorosilane, is added along with an organic amine such as triethylamine, trimethylamine, 4-dimethylaminopyridine or imidazole. Ordinarily imidazole is the preferred base. The reaction mixture is stirred for about one hour to about 24 hours, typically at room temperature, after which time the product is isolated from the reaction 15 broth in a manner well known to one of ordinary skill in the art.

The macrolide, now protected at the C-4" position, can be coupled with a sugar halide of formula (V) as described hereinabove. The product of such a coupling reaction is a derivative of a compound of formula (I) with a sugar derivative attached by way of oxygen to the C-14 position and with a protected C-4" position. The C-4" position 20 can be deprotected to afford the free hydroxy compound by employing standard methods of organic chemistry well known to one of ordinary skill in the art. Typically, to remove a preferred silyl ether protecting group, the C-4"-silyl protected compound of formula (I) is dissolved in a acetonitrile or reaction inert solvent such as an ether solvent such as tetrahydrofuran or diethyl ether at a temperature of about 0°C to 30°C 25 and is treated with a fluoride source such as hydrogen fluoride or tetra-N-butylammonium fluoride. The reaction is stirred for about one hour to about 24 hours and the product is then isolated by employing standard methods of organic chemistry well known to one of ordinary skill in the art.

To prepare the compounds of the invention of formula (I) wherein A and B are 30 taken separately and are each H (hereinafter referred to as the C-2 desoxo macrolide), a compound of formula (I) wherein A and B are taken together and are =O is reduced using standard conditions for the reduction of α -ketoamides. This reduction procedure selectively reduces the carbonyl adjacent to the amide without affecting other carbonyls

in the molecule. Generally the C-2 oxo macrolide of formula (I) is dissolved in a reaction inert solvent or mixture of solvents and hydrogen sulfide gas is bubbled through the mixture for 6-24 hours at room temperature. For convenience, the gas is generally bubbled through the reaction mixture overnight. Suitable reaction inert solvents for this
5 reaction include, but are not limited to, organic bases such as diethylamine, triethylamine, dimethylamine, trimethylamine, piperidine, morpholine and aniline; dipolar aprotic solvents such as N,N-dimethylformamide, dimethylsulfoxide and N-methylpyrrolidone; and alcoholic solvents such as methanol, ethanol and propanol. A combination of two or more of these solvents is sometimes used to achieve optimum
10 yield or to affect the course of reduction. For example, the macrolide wherein A is H and B is OH is prepared by using methanol as solvent. A particularly preferred solvent system for providing the C-2 desoxo macrolide is pyridine and N,N-dimethylformamide in equal amounts. When the reaction is completed, the product is isolated using the standard techniques of organic chemistry as would be understood by one of ordinary
15 skill in the art.

Alternatively, the macrolide of formula (III) or (IV) can be reduced prior to glycosylation, using the foregoing procedure. Following reduction, the macrolide can be glycosylated as recited hereinabove.

To prepare compounds of the invention of formula (I) wherein the dotted line
20 represents a bond and R² is hydrogen, the compound of formula (I) wherein R² is -OH and the dotted line represents no bond (hereinafter referred to as the β-hydroxy ketone) is dehydrated as disclosed in European Patent Application No. 323042. Generally the β-hydroxy ketone is dissolved in a reaction inert solvent containing a catalytic amount of an organic acid. Suitable reaction inert solvents are
25 aromatic solvents such as benzene, toluene, xylene and the like, with toluene being preferred. The organic acid is generally selected from such acids as toluenesulfonic acid, camphorsulfonic acid and the like with toluenesulfonic acid being preferred. The reaction mixture is heated at about 50°C to about 120°C for about five minutes to about one hour. Generally steam bath temperatures (about 100°C) are preferred and
30 five minutes is generally sufficient for complete reaction. The reaction product is isolated according to methods well understood by one of ordinary skill in the art. The reaction is generally carried out on compounds which have already been glycosylated.

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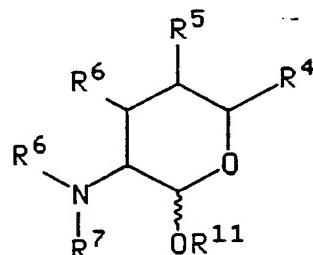
To prepare compounds of the invention of formula (I) wherein R⁵ and R⁶ are hydroxy and R⁴ is hydroxymethyl, a compound of formula (I) wherein R⁵ and R⁶ are acetoxy and R⁴ is acetoxyethyl is deacetylated using standard conditions known to one of ordinary skill in the art as recited hereinbelow. This selective deacetylation does

5 not affect any amides which are present and is readily accomplished by the addition of an alkoxide base to a solution of the material to be deacetylated in an alcoholic solvent at 0°C. Generally a catalytic amount, such as 0.01 equivalents, of base is used. Usually the alkoxide base of the particular alcoholic solvent in use is preferred.

10 Most preferred, for its ease of use and reactivity, is the system wherein methanol is the solvent and sodium methoxide is the base. Isolation of the product is achieved via standard methods well known to those of ordinary skill in the art.

The sugar halide derivatives of formula (V) are conveniently prepared, when not readily available, from the readily available 2-amino sugar of formulae (VI) wherein R¹¹ is H, (C₁-C₄)alkyl.

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(VI)

or (C₂-C₄)alkanoyl by employing standard methods of halogenation well known to one of ordinary skill in the art.

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Bromination is the method of choice; chlorination may also be employed in certain cases. Bromination is effected by dissolving a 1-hydroxy, alkoxy or alkanoyloxy sugar derivative of formula (VI) in an organic acid solvent such as acetic acid. When the sugar is a 1-hydroxy or 1-alkoxy sugar, one to ten equivalents of acetic anhydride are generally added. The preferred substrates are 1-acetoxy sugar derivatives. The

30 reaction mixture is cooled so that the temperature falls within the range of about -20°C to about 0°C. The generally preferred temperature is about 0°C. The cooled reaction mixture is treated with a solution of hydrobromic acid in the acidic solvent. Generally a large excess, such as 10-40 molar equivalents, of hydrobromic acid is employed. The

reaction mixture is warmed to room temperature and stirred until the reaction is complete. Generally, for convenience, the reaction mixture is left stirring overnight. The isolation of the brominated product is achieved in a straightforward manner well known to one of ordinary skill in the art. Often this merely involves removing the solvent *in vacuo*. Occasionally, to more fully effect solvent removal, a cosolvent, such as toluene, which azeotropes the reaction solvent is utilized. Further purification is sometimes achieved by the use of column chromatography.

- To prepare a compound of formula (VI) wherein R⁷ and R⁸ are each (C₁-C₄)alkyl, the compound of formula (VI) wherein R⁷ and R⁸ are each H is reacted under standard alkylation conditions known to one of ordinary skill in the art. Similarly, to prepare a compound of formula (VI) wherein R⁷ is H and R⁸ is -COR⁹, -CO₂R⁹, COCH₂R⁹ or -SO₂R⁹, a compound of formula (VI) wherein R⁷ and R⁸ are each H is reacted employing standard amino-acylating or -sulfonating techniques. Thus, a compound of formula (VI) wherein R⁷ and R⁸ are each H is reacted with an excess of an acylating or sulfonating reagent such as, but not limited to, acetic anhydride, trifluoroacetic anhydride, (C₁-C₆)alkanoyl chloride, (C₃-C₆)cycloalkanoyl chloride, benzoyl chloride, crotonyl chloride, nicotinoyl chloride, isonicotinoyl chloride, picolinoyl chloride, thiophene carbonyl chloride, furfuryl chloride or an R⁹-substituted sulfonyl chloride in a reaction inert solvent. Suitable reaction inert solvents for a reaction of this type include chlorinated solvents such as chloroform, methylene chloride and ethylene dichloride; aromatic solvents such as benzene, toluene and xylene; and dipolar aprotic solvents such as N,N-dimethylformamide, dimethylsulfoxide and N-methylpyrrolidone. Preferred are chlorinated solvents and especially preferred is methylene chloride. When the reaction is carried out using the acid addition salt form of the aminosugar, it is necessary to add a base as a proton scavenger. Generally it is sufficient to employ a weak base such as pyridine or an organic amine such as diethylamine, triethylamine, trimethylamine or piperidine. The most preferred base for this particular reaction is pyridine. Isolation of the product using standard techniques known to those skilled in the art yields the acyl derivative of the amino sugar.
- To prepare the aminosugar derivatives of formula (VI) wherein R⁴ is -CO₂R⁹ or -CO₂H, the 1-hydroxy group of an aminosugar of formula (VI) wherein R⁷ and R⁸ are each H, R⁴ is -CH₂OH and R⁵ and R⁶ are each -OH, is protected utilizing standard protection techniques known to one of ordinary skill in the art. Typically a benzyl group

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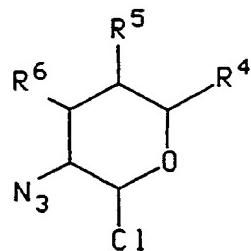
or the like is used as the protecting group. The protected aminosugar of formula (VI) wherein R⁷ and R⁸ are each H is then N-alkylated, N-acylated or N-sulfonated as described hereinabove. The N-substituted, 1-protected aminosugar of formula (VI) wherein R⁴ is -CH₂OH and R⁵ and R⁶ are each -OH is then oxidized utilizing standard sugar oxidation chemistry known to one of ordinary skill in the art. Typically, the aminosugar of formula (VI) wherein R⁵ and R⁶ are each -OH; R⁴ is -CH₂OH; R⁷ is H; R⁸ is -COR⁹, -CO₂R⁹, -CO₂CH₂R⁹ or -SO₂R⁹; and R¹⁰ is a protecting group is dissolved in a reaction inert solvent such as benzene, acetonitrile or ethyl acetate and treated with a catalytic amount of platinum oxide. Oxygen gas is bubbled through the reaction mixture for about 2 hours to about 24 hours at room temperature. The uronic acid derivative is isolated from the reaction broth utilizing standard techniques known to one of ordinary skill in the art.

To prepare the compounds of formula (VI) wherein R⁵ and R⁶ are each -OCOR⁹ or -OCO₂R⁹, a compound of formula VI wherein R⁵ and R⁶ are each -OH is reacted under the standard acylation conditions of organic chemistry. Typically a compound of formula (VI) wherein R⁵ and R⁶ are each -OH is reacted with a suitable acylating agent such as, but not limited to, acetic anhydride in a reaction inert solvent such as acetic acid at about 0°C to about 25°C. The product is isolated utilizing standard techniques of chemistry known to one of ordinary skill in the art.

20 To prepare the compounds of formula (VI) wherein R⁴ is -CH₂OCOR⁹ or -CH₂OCO₂R⁹ and R⁵ and R⁶ are each -OCOR⁹ or -OCO₂R⁹, the procedure of the preceding paragraph is utilized, starting with a compound of formula (VI) wherein R⁴ is -CH₂OH and R⁵ and R⁶ are each -OH.

Alternatively, compounds of formula (I) can be prepared by employing the coupling (glycosylation) reaction recited hereinabove but substituting an azido-25 substituted sugar derivative of the formula

30



(VII)

for the sugar halide of formula (V). Azido compounds of this formula are prepared using the method described by Lemieux et al. [Canadian Journal of Chemistry, 57, 1244-51 (1979)].

- The macrolide thus obtained after coupling with the azidosugarchloride is
- 5 hydrogenated and acylated *in situ* to afford macrolides of formula (I) wherein R⁴ is -CH₂OCOR⁹ or -CH₂OCO₂R⁹, R⁵ and R⁶ are each -OCOR⁹ or -OCO₂R⁹, R⁷ is H and R⁸ is -COR⁹ or -CO₂R⁹.

The hydrogenation is generally carried out by stirring or shaking the material to be hydrogenated (substrate) in a reaction inert solvent in the presence of a noble metal catalyst. The reaction is generally performed at a temperature of about 0°C to 60°C and preferably at about 25°C. The hydrogen pressure is generally maintained in the range of about atmospheric pressure to about 60 PSI, with about 20 to about 50 PSI being more preferred. Most preferred is a pressure of 50 PSI. The reaction inert solvent is selected from such solvents as diethyl ether, tetrahydrofuran, dioxane, 1,2-dimethoxyethane, dimethylformamide, ethanol, methanol, formic acid, acetic acid and propionic acid. An especially preferred solvent for this particular hydrogenation is acetic acid. The preferred noble metal catalysts for use in this reaction are palladium, platinum, rhodium and nickel. Especially preferred for its reactivity is palladium. The catalyst is conveniently supported on an inert medium such as carbon, and the catalyst is usually present in an amount from 0.01 weight-percent to about 25 weight-percent. Especially preferred is the range of 0.1 percent to 10 weight-percent, based on the azido compound's weight. The reaction is generally carried out in a sealed flask, e.g., Parr bottle attached to a Parr Shaker Apparatus for introducing the hydrogen and maintaining the desired pressure. When the reaction is carried out under these conditions, it is usually completed within a few hours, e.g., from about 2 to about 24 hours.

When the hydrogenation is complete, the reaction flask is removed from the Parr Shaker and its contents are treated with enough acetic anhydride to ensure complete reaction with the newly-formed amine. The reaction vessel is swirled for a few minutes, e.g., 3 to 30 minutes, at which time the reaction mixture is filtered through Celite and charcoal. The filtrate is purified according to standard methods of organic chemistry to afford the desired macrolide analog.

When the compounds of formula (I) of the present invention are acidic, as when R⁴ is -CO₂H, the invention also embraces pharmaceutically acceptable salts of said compounds of formula (I).

Typical pharmaceutically acceptable cationic salts for such use include alkali metal salts (e.g., sodium and potassium), alkaline earth metal salts (e.g., magnesium and calcium), aluminum salts, ammonium salts and salts with organic amines such as benzathine (N,N'-dibenzylethylenediamine), choline, diethanolamine, ethylenediamine, megilumine(N-methylglucamine),benethamine(N-benzylphenethylamine),diethylamine, piperazine, tromethamine (2-amino-2-hydroxymethyl-1,3-propanediol) and procaine. An especially preferred such salt is the sodium salt.

The pharmaceutically acceptable cationic salts of the present invention are readily prepared by reacting the acid forms with an appropriate base, usually one equivalent, in a co-solvent. Typical bases are sodium hydroxide, sodium methoxide, magnesium hydroxide, calcium hydroxide, benzathine, choline, diethanolamine, piperazine and tromethamine. The salt is isolated by concentration to dryness or by addition of a non-solvent. In many cases, salts are preferably prepared by mixing a solution of the acid with a solution of a different salt of the cation (sodium or potassium ethylhexanoate, magnesium oleate), employing a solvent (e.g., ethyl acetate) from which the desired cationic salt precipitates, or can be otherwise isolated by concentration and/or addition of a non-solvent.

When the compounds of formula (I) of this invention are basic, as when R⁷ and R⁸ are each H or (C₁-C₄)alkyl or when R⁷ is H and R⁸ is (C₁-C₄)alkyl, the invention also embraces pharmaceutically acceptable acid addition salts of said compounds of formula (I).

Typical pharmaceutically acceptable acid addition salts include hydrochloride, hydrobromide, sulfate, hydrogen sulfate, phosphate, hydrogen phosphate, dihydrogen phosphate, acetate, succinate, citrate, mesylate (methanesulfonate) and tosylate (p-toluenesulfonate). These salts are readily prepared by reacting the base forms with the appropriate acid. When the salt is of a monobasic acid (e.g., the hydrochloride, hydrobromide, p-toluenesulfonate or acetate), the hydrogen form of a dibasic acid (e.g., the hydrogen sulfate or succinate) or the dihydrogen form of a tribasic acid (e.g., the dihydrogen phosphate or citrate), at least one molar equivalent and usually a molar excess of the acid is employed. However, when such salts as the sulfate, the

hemisuccinate, the hydrogen phosphate or the phosphate are desired, the appropriate and exact chemical equivalents will generally be used. The free base and the acid are usually combined in a co-solvent from which the desired salt precipitates or can be otherwise isolated by concentration and/or addition of a non-solvent.

5 With respect to the macrolides of formula (I) of this invention, it is to be understood that there are conformer(s) or stereoisomeric forms such as optical and geometrical isomers due to asymmetric carbon atom(s) and double bond(s), and such isomers are also included within the scope of this invention.

The compounds of formula (I) thus prepared are useful in the treatment of
10 resistance to transplantation and autoimmune diseases such as rheumatoid arthritis or psoriasis. In the treatment of resistance to transplantation, a compound of formula (I) may be used either prophylactically or in response to an adverse reaction by the human subject to a transplanted organ or tissue. When used prophylactically, a compound of formula (I) is administered to the patient or to the tissue or organ to be
15 transplanted in advance of the transplantation operation. Prophylactic treatment may also include administration of the medication after the transplantation operation but before any signs of adverse reaction to transplantation are observed. When administered in response to an adverse reaction, a compound of formula (I) is administered directly to the patient in order to treat said resistance to transplantation after
20 outward signs of the resistance have been manifested.

For use in the treatment of resistance to transplantation and autoimmune diseases such as rheumatoid arthritis or psoriasis in a mammal, including man, a compound of formula (I) is formulated into a suitable pharmaceutical composition containing a disease treating effective amount. Depending upon the potency of the
25 particular compound of formula (I) being administered, about 0.05 mg/kg of body weight per day to about 30 mg/kg of body weight per day, in single or multiple daily doses, is administered to the mammal being treated. A more preferred range is 0.1 mg/kg of body weight per day to about 20 mg/kg of body weight per day, although in particular cases, at the discretion of the attending physician, doses outside the
30 broader range may be required. The preferred route of administration is generally oral, but parenteral administration (e.g., intravenous, intramuscular, subcutaneous or intramedullary) will be preferred in special cases such as where oral administration is inappropriate to the instant target or where the patient is unable for various reasons to

ingest the drug. Topical administration may also be indicated, as where the patient is suffering from a skin disease such as psoriasis or whenever the medication is best applied to the surface of a tissue or organ as determined by the attending physician.

The compounds of formula (I) thus prepared are also useful in the treatment of infections caused by fungi. For use in the treatment of said fungal infections in a mammal, including man, a compound of formula (I) is formulated into a pharmaceutical composition containing a disease treating effective amount. Depending upon the potency of the particular compound of formula (I) being administered, about 0.05 mg/kg of body weight per day to about 30 mg/kg of body weight per day, in single or multiple daily doses, is the amount administered to the mammal being treated. A more preferred range is 0.1 mg/kg of body weight per day to about 20 mg/kg of body weight per day, although in particular cases, at the discretion of the attending physician, doses outside the broader range may be required. The preferred route of administration is generally oral, but parenteral administration (e.g., intravenous, intramuscular, subcutaneous or intramedullary) will be preferred in special cases such as where oral administration is inappropriate to the instant target or where the patient is unable for various reasons to ingest the drug. Topical administration may also be indicated whenever the medication is best applied to the surface of a tissue or organ as determined by the attending physician.

The compounds of the present invention are generally administered in the form of a pharmaceutical composition comprising at least one of the compounds of formula (I) together with a pharmaceutically acceptable vehicle or diluent. Such compositions are generally formulated in a conventional manner utilizing solid or liquid vehicles or diluents as appropriate to the mode of administration: for oral administration, in the form of tablets, hard or soft gelatin capsules, suspensions, granules, powders and the like; for parenteral administration, in the form of injectable solutions or suspensions and the like; and for topical administration, in the form of solutions, lotions, ointments, salves and the like.

The utility of the compounds of the present invention as medical agents in the treatment of resistance to transplantation and autoimmune diseases such as rheumatoid arthritis or psoriasis is demonstrated by the activity of said compounds in the biological screen described hereinbelow. Said biological screen also provides a means whereby the activities of the compounds of formula (I) can be compared with

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the activities of other known compounds. The results of these comparisons are useful for determining dosage levels in mammals, including man, for the treatment of resistance to transplantation and autoimmune diseases such as rheumatoid arthritis and psoriasis.

5 The human mixed lymphocyte reaction (MLR) is used to generate an immune response *in vitro* which is measured via ^3H -thymidine uptake. This screen uses peripheral blood mononuclear cells in a modified two-way MLR. To ensure disparity of HLA type D antigens and therefore maximize stimulation, a pool of frozen donor cells is used as the stimulator population; freshly isolated cells are used as the responder
10 population.

Freshly drawn mononuclear cells are suspended in RPMI-1640 enriched with: 0.5% MEM non-essential amino acids (100x) solution, 1% L-glutamine (200 mM), 1% MEM vitamins (100x), 1% penicillin streptomycin solution (10,000 units/mL) and 15% heat-inactivated human AB serum (NABI). The cells are counted and the concentration
15 is adjusted to 5×10^5 cells/mL. The solution is then transferred to round bottom 96 well plates in 100 μL /well quantities. These plates now contain the responder cells.

The stimulator cells are prepared by pooling the mononuclear cells collected from several different individuals. The cells are suspended in 90% human AB serum and 10% DMSO such that the cell count is 2×10^7 cells/mL. The cells are stored in
20 liquid nitrogen. For an MLR, the viable cells are diluted to 5×10^5 cells/mL, and 100 μL /well is added to the plates containing the responder cells. To each well, containing a mixture of responder cells and stimulator cells, is added 50 μL of compound solution. Triplicate wells are run for each dose. The plates are incubated at 37°C under an atmosphere of 5% CO₂ and are humidified for five days. To each well is
25 added 1 μCi of ^3H -thymidine and incubation is continued for another eighteen hours. The cells are harvested using the LKB Beta Plate system.

The percent inhibition of stimulated control is obtained using the following equation:

30 % Inhibition =
$$\left[100 - \left(\frac{\text{avg. cpm of drug}}{\text{avg. cpm of stimulated control}} \right) \right] \times 100$$

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The abbreviation cpm is defined as counts per minute. RPMI-1640 is a tissue culture medium which is available from Sigma.

Activity in the MLR screen recited above is indicative of usefulness of the active compound in the treatment of resistance to transplantation and autoimmune diseases
5 such as rheumatoid arthritis and psoriasis.

Antimicrobial activities of the macrolides of the present invention against various fungi are determined by a serial agar dilution method in a Sabouraud agar. Minimum inhibitory concentrations (MIC) are obtained after incubation for 24 hours at 30°C.

The present invention is illustrated by the following examples. However, it
10 should be understood that the invention is not limited to the specific details of these examples. All reactions are conducted under an inert atmosphere, such as nitrogen, unless otherwise specified. The abbreviations THF, DMSO, DAST, DMAP and Ac, where used, refer to tetrahydrofuran, dimethyl sulfoxide, dimethylamino sulfurtrifluoride, 4-dimethylaminopyridine and acetyl, respectively. The sugar halides were purchased
15 from a reliable vendor such as Sigma or Aldrich, as were the sugars, unless specifically mentioned. Anhydrous solvents were used, anhydrous being defined as substantially free from water.

The expression "reaction inert solvent," where used hereinabove, refers to any solvent which does not interact with starting materials, reagents, intermediates or
20 products in a manner which adversely affects the yield of the desired product.

Terms or acronyms which appear in Preparations 1 and 2 are described in further detail hereinbelow.

PYEA agar is prepared by dissolving Difco maltose (10 g), Difco yeast extract (4 g), dextrose (4 g), Difco agar (15 g) and fresh coconut milk (50 mL) in enough
25 deionized water to yield a one liter solution; and the solution is adjusted to pH 7.3 with 1N NaOH.

ATCC 172 medium is prepared by dissolving glucose (10 g), soluble starch (20 g), yeast extract (5 g), NZ-amine A (Difco, 5 g) and calcium carbonate (1 g) in enough deionized water to yield a one liter solution; and the solution is adjusted to
30 pH 7.0 with 1N KOH.

JDYTT medium is prepared by dissolving cerelose (10 g), corn starch (5 g), corn steep liquor (5 g), NZ-amine YTT (5 g), cobalt chloride (0.002 g) and calcium carbonate

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(3 g) in enough deionized water to yield a one liter solution; and the solution is adjusted to pH 7.2 with 1N NaOH.

NZ-amine A and NA-amine YTT are available from Difco, as are most of the ingredients of the above media.

- 5 In the MLR protocol provided hereinabove, RPMI-1640 is a standard medium for MLR studies; MEM is defined as "minimum essential media"; and NABI is a supplier.

EXAMPLE 1

17-Ethyl-1,14-dihydroxy-12-[2'-(4''-(2'''-deoxy-2'''-trifluoroacetamido-3'',4'',6'''-tri-O-acetyl- β -D-gluco-pyranosyloxy)-3'''-methoxycyclohexyl)-1'-methylvinyl]-23,25-dimethoxy-
5 13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.O^{4,9}]-octacos-18-ene-2,3,10,16-tetraone.

To a stirred slurry of the title compound of Preparation 1 (2.48 g), the title compound of Preparation 3 (2.9 g) and 4 \AA Molecular Sieves (crushed, 5.0 g) in methylene chloride (anhydrous, 150 mL) at -78°C was added silver carbonate (5.15 g) followed by silver triflate (0.83 g). The reaction mixture was allowed to warm to room
10 temperature over 8 hours and was then stirred for an additional 5 hours. The resultant tan slurry was filtered through Celite® and the filtrate was evaporated *in vacuo*. The residue was purified on silica gel, eluting with hexane/ethyl acetate (2/1) to afford the product (0.93 g, 25%).

13C NMR (300 MHz, CDCl₃) (major rotamer): δ 213.36, 197.1, 192.82, 170.94,
15 170.77, 169.44, 169.03, 164.92 and 138.85. Mass spectrum (FAB): 1197.2 (molecular ion + Na⁺).

EXAMPLES 2 and 3

Using substantially the same procedure as recited in Example 1 but substituting one molar equivalent of the appropriate sugar halide for the title compound of Preparation 3,
20 the following compounds were prepared.

2. 17-Ethyl-1,14-dihydroxy-12-[2'-(4''-(2'''-deoxy-2'''-acetamido-3'',4'',6'''-tri-O-acetyl- β -D-glucopyrano-syloxy)-3'''-methoxycyclohexyl)-1'-methylvinyl]-23,25-dimethoxy-
13,19,21,27-tetramethyl-11,28-dioxa-4-azatri-cyclo[22.3.1.O^{4,9}]-octacos-18-ene-2,3,10,16-tetraone.

25 13C NMR (500 MHz, CD₃COCD₃) (major rotamer): δ 211.94, 197.93, 170.75, 170.478, 170.21, 170.013, 169.80, 166.1, 138.88, 132.80 and 132.21. Mass spectrum (FAB): 1143.5 (molecular ion + Na⁺).

30 3. 17-Ethyl-1,14-dihydroxy-12-[2'-(4''-(2'''-deoxy-2'''-azido-3'',4'',6'''-tri-O-acetyl- β -D-galactopyranosyloxy)-3'''-methoxycyclohexyl)-1'-methylvinyl]-23,25-dimethoxy-
13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.O^{4,9}]-octacos-18-ene-2,3,10,16-tetraone.

Mass spectrum (FAB): 1127 (molecular ion + Na⁺).

EXAMPLE 4

- 5 17-Ethyl-1,14-dihydroxy-12-[2'-(4''-(2'''-deoxy-2'''-acetamido-3'',4'',6'''-tri-O-acetyl- β -D-galactopyrano-syloxy)-3'''-methoxycyclohexyl)-1'-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatri-cyclo[22.3.1.O^{4,9}]octacos-18-ene-2,3,10,16-tetraone.

The title compound of Example 3 (1 g) and 5% palladium-on-carbon (100 mg) were diluted with acetic acid (10 mL) and the reaction mixture was hydrogenated at 50 PSI of hydrogen pressure and room temperature for 16 hours. The reaction mixture was treated with 2 mL of acetic anhydride and swirled for 5 minutes. The reaction 10 mixture was filtered through Celite and charcoal, concentrated *in vacuo* and chromatographed on silica gel [eluting first with ethyl acetate/hexane (1/1) and then with ethyl acetate/hexane (2/1)] to afford mg of the title compound of this Example.

Mass spectrum (FAB): 1143.3 (molecular ion + Na⁺).

EXAMPLE 5

- 15 17-Ethyl-1,14-dihydroxy-12-[2'-(4''-(2'''-deoxy-2'''-acetamido-3'',4'',6'''-tri-O-acetyl- β -D-glucopyrano-syloxy)-3'''-methoxycyclohexyl)-1'-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatri-cyclo[22.3.1.O^{4,9}]octacos-18-ene-3,10,16-trione.

The title compound of Example 2 (1.0 g) was dissolved in DMF (15 mL) and pyridine (15 mL) and hydrogen sulfide (H₂S) gas was bubbled through the reaction 20 mixture overnight at room temperature. Excess H₂S was prevented from escaping into the atmosphere by means of a Chlorox trap. After 16 hours the reaction mixture was diluted with toluene (50 mL) and washed with brine (50 mL). The solvent was dried with MgSO₄ and the solvent was removed *in vacuo*. The residue was passed through a pad of silica gel and eluted with ethyl acetate/hexane (2/1) to afford 490 mg (50%) of the title 25 compound of this Example.

¹³C NMR (300 MHz, CD₂Cl₂) (major rotamer): δ 215.14, 174.08, 171.74, 170.48, 170.39, 169.93, 169.36, 169.137, 140.76, 132.04, 128.45 and 122.23. Mass spectrum (FAB): 1129.7 (molecular ion + Na⁺).

EXAMPLE 6

- 30 17-Ethyl-1,14-dihydroxy-12-[2'-(4''-(2'''-deoxy-2'''-trifluoroacetamido- β -D-glucopyranosyloxy)-3'''-methoxycyclohexyl)-1'-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.O^{4,9}]octacos-18-ene-2,3,10,16-tetraone.

The title compound of Example 1 (1.0 g) was dissolved in methanol (100 mL) and treated with sodium methoxide (20 mg). The reaction mixture was stirred at 0°C 35 for 8 hours and then was treated with 1 drop of acetic acid from a disposable pipet.

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The solvent was removed *in vacuo* and the residue was purified on silica gel, eluting with THF, to afford 610 mg of a foam (66%).

EXAMPLES 7 and 8

- Using substantially the same procedure as recited in Example 6, but substituting
- 5 1 molar equivalent of one of the products of Examples 2 to 5 for the product of Example 1, the following compounds were prepared.
7. 17-Ethyl-1,14-dihydroxy-12-[2'-(4"- (2""-deoxy-2""-acetamido- β -D-glucopyranosyloxy)-3"-methoxycyclohexyl]-1'-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.O^{4,9}]octacos-18-ene-2,3,10,16-tetraone.
- 10 ¹³C NMR (300 MHz, CD₃OD) (major rotamer): δ 212.02, 196.8, 172.26, 169.29 and 166.08. Mass spectrum (FAB): 1017.6 (molecular ion + Na⁺).
8. 17-Ethyl-1,14-dihydroxy-12-[2'-(4"- (2""-deoxy-2""-acetamido- β -D-glucopyranosyloxy)-3"-methoxycyclohexyl]-1'-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.O^{4,9}]octacos-18-ene-3,10,16-trione.
- 15 ¹³C NMR (300 MHz, CD₂Cl₂) (major rotamer): δ 215.0, 174.1, 172.6, 169.5, 141.1.

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PREPARATION 1

17-Ethyl-1,14-dihydroxy-12-[2'-(4"-hydroxy-3"-methoxy-cyclohexyl)-1'-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.O^{4,9}]-octacos-18-ene-2,3,10,16-tetraone

5 *Streptomyces hygroscopicus* subsp. *ascomyceticus* culture ATCC 14891 was carried on PYEA agar slants (10 g/L of Difco maltose, 4 g/L of Difco yeast extract, 4 g/L of dextrose, 15 g/L of Difco agar and 50 mL of fresh coconut milk which was diluted up to one liter with deionized water, then adjusted to pH 7.3 with 1N NaOH). The preparation was incubated for 10 to 12 days at 28°C, and the spores were then
10 transferred to sterile 1x6 shake tubes containing 10 mL of ATCC 172 medium (10 g/L of glucose, 20 g/L of soluble starch, 5 g/L of yeast extract, 5 g/L of NZ-amine A and 1 g/L of calcium carbonate. The pH was adjusted to 7.0 with 1N KOH). The tubes were incubated at 28°C and shaken on a rotary shaker at about 150 to 200 cycles/minute. After 4 to 5 days the broth was diluted to 40% with glycerol and
15 ATCC 172 medium and then transferred aseptically to cryotubes. Each tube was charged with 1/2 mL of broth. The tubes were frozen at -80°C during storage.

The tubes from the ultra cold stock were used as seed inoculum for the preparation of inoculum flasks, one tube per 50 mL of sterile JDYTT medium in 300 mL shake flasks. The composition of the JDYTT medium was 10 g/L of cereose,
20 5 g/L of NZ-amine YTT, 0.002 g/L of cobalt chloride and 3 g/L of calcium carbonate. The pH of the JDYTT medium was adjusted to pH 7.2 with 1N NaOH. The shake flasks were shaken and incubated on a rotary shaker at about 150-200 cycles/minute and 28°C.

Two mL of an about 3 to 5 day-old shake flask inoculum was used to
25 inoculate the second stage flask inoculum containing 80 mL of JDYTT medium in a 3L jar fermenter. The fermenter medium was 45 g/L of corn starch, 10 g/L of corn steep liquor, 10 g/L of amber or Baker dried yeast, 3 g/L of calcium carbonate and 0.005 g/L of cobalt chloride. The pH was adjusted to about 6.4 to 6.8 with 1N NaOH. One mL of antifoam P-2000 was added to the jar fermenters together with 100 mL of
30 soya bean oil. The pH was adjusted to about 6.4 to 6.8 with 1N NaOH and the material was agitated at 1700 rpm. The temperature was maintained at 28°C and sterile air was sparged through the medium at the rate of one volume per volume per minute.

After inoculation, sterile soya bean oil was used to control foaming. In longer fermentations, and, depending on media used, the sugar content can be monitored and

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sugar feeds used at 40, 60 and 90 hours to maintain the reducing sugar level at or above 0.05%. The fermentation was run for 46 hours.

Using standard methods of thin-layer chromatography and HPLC, the fermentation broth was monitored and relative potency was calculated.

5 The product was found primarily in the mycelium, but workup of the whole broth is preferred. Thus, after the fermentation has run its course, the whole broth was extracted twice with one-third to one-half of its volume of methylisobutylketone (MIBK). The layers were separated by means of a DeLaval separator or a Podbielnack extractor. The solvent layer was clarified and concentrated first in a vacuum pan and then in a
10 rotary evaporator. The concentrate was subjected to four tube counter current distribution in 20 liter carbuoys using 10 liter top layer and 1 liter bottom layer per carbuoy of a heptane/acetonitrile 10/1 system. The active bottom layers were collected, combined and concentrated. The material was further purified *via* filtration through Florisil (washing with hexane, hexane/methylene chloride and methylene chloride,
15 successively, with a gradual increase in methylene chloride). Most of the activity was found in the methylene chloride fractions. These were combined and concentrated. A second filtration step was performed, this time through silica gel (washing with heptane, methylene chloride, methylene chloride/ethyl acetate and ethyl acetate). The activity was mostly found in the fractions containing amethylene chloride/ethyl acetate
20 mixture and the fractions containing only ethyl acetate. These were combined and concentrated, redissolved in methylene chloride and treated with DARCO G60. The sample was then divided into 12 to 15 g portions and each sample was further chromatographed on a Prep 500 liquid chromatograph using silica gel columns and eluting using a linear gradient beginning with 100% methylene chloride and ending with
25 100% ethyl acetate. The active cuts were combined, concentrated and chromatographed on a Prep 500, using reversed phase (¹⁸C) silica gel and eluting with a linear gradient beginning with acetone and ending with 100% water. Clean product was obtained as the last component isolated off the column.

The active fractions in the foregoing fermentation procedure were determined
30 using the following bioassay.

A 12.5 mm disc was applied directly to the agar surface. *Candida albicans* ATCC 14053, *Saccharomyces pastorianus* FD3737 and a sensitive strain of *Byssochlamys fulva* FM 10,300(S) and FM 10,464(R) were used. The *Candida* and

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Saccharomyces plates were incubated at 37°C for 18 hours, then the plates were examined for activity. The *Byssochlamys* plates were incubated at 28°C and read after 18 hours. Plates containing only FK506 and FK520 (CP-105051) were active against the *Byssochlamys* strain. Impure fractions (containing nigericin) were active against the other strains as well.

An HPLC method for determining the purity of the fractions was also used. The method entailed using a Dupont Zorbax CN column (4.6 mm x 25 cm) and an isocratic system composed of 55/45 water/acetonitrile and a flow rate of one mL/min. Detection was accomplished at 214 nm. The broth sample (20 mL) was mixed with MIBK (20 mL) and shaken for about 4 to 5 minutes. The layers were separated and the solvent was concentrated to near dryness. The residue was taken up in 1 mL of neat acetonitrile and a 5 µL sample was injected into the HPLC. The retention time for FK520 is approximately 12.7 minutes under these conditions.

PREPARATION 2

15 17-Allyl-1,14-dihydroxy-12-[2'-(4"-hydroxy-3"-methoxy-cyclohexyl)-1'-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.O^{4,9}]octacos-18-ene-2,3,10,16-tetraone

Streptomyces tsukubaensis No. 9993 FERM BP-927 was carried on PYEA agar slants (10 g/L of Difco maltose, 4 g/L of Difco yeast extract, 4 g/L of dextrose, 15 g/L of Difco agar and 50 mL of fresh coconut milk which was diluted up to one liter with deionized water, then adjusted to pH 7.3 with 1N NaOH). The preparation was incubated for 10 to 12 days at 28°C, and the spores were then transferred to sterile 1x6 shake tubes containing 10 mL of ATCC 172 medium (10 g/L of glucose, 20 g/L of soluble starch, 5 g/L of yeast extract, 5 g/L of NZ-amine A and 1 g/L of calcium carbonate. The pH was adjusted to 7.0 with 1N KOH). The tubes were incubated at 28°C and shaken on a rotary shaker at about 150 to 200 cycles/minute. After 4 to 5 days the broth was diluted to 40% with glycerol and ATCC 172 medium and then transferred aseptically to cryotubes. Each tube was charged with 1/2 mL of broth. The tubes were frozen at -80°C during storage.

30 The tubes from the ultra cold stock were used as seed inoculum for the preparation of inoculum flasks, one tube per 50 mL of sterile JDYTT medium in 300 mL shake flasks. The composition of the JDYTT medium was 10 g/L of cerelose, 5 g/L of NZ-amine YTT, 0.002 g/L of cobalt chloride and 3 g/L of calcium carbonate. The pH of the JDYTT medium was adjusted to pH 7.2 with 1N NaOH. The shake flasks

were shaken and incubated on a rotary shaker at about 150-200 cycles/minute and 28°C.

Two mL of an about 3 to 5 day-old shake flask innoculum was used to inoculate the second stage flask innoculum containing 80 mL of JDYTT medium in a 5 3L jar fermenter. The fermenter medium was 45 g/L of corn starch, 10 g/L of corn steep liquor, 10 g/L of amber or Baker dried yeast, 3 g/L of calcium carbonate and 0.005 g/L of cobalt chloride. The pH was adjusted to about 6.4 to 6.8 with 1N NaOH. One mL of antifoam P-2000 was added to the jar fermenters togetherwith 100 mL of soya bean oil. The pH was adjusted to about 6.4 to 6.8 with 1N NaOH and the material 10 was agitated at 1700 rpm. The temperature was maintained at 28°C and sterile air was sparged through the medium at the rate of one volume per volume per minute.

After inoculation, sterile soya bean oil was used to control foaming. In longer fermentations, and, depending on media used, the sugar content can be monitored and sugar feeds used at 40, 60 and 90 hours to maintain the reducing sugar level at or 15 above 0.05%. The fermentation was run for 46 hours.

Using standard methods of thin-layer chromatography and HPLC, the fermentation broth was monitored and relative potency was calculated.

The fermenters were stopped and extracted twice with ½ its volume of methylisobutylketone (MIBK). The solvent layer was separated by aspiration and 20 concentration *in vacuo* to a viscous oil. The oil was triturated with hexane, diethyl ether and methylene chloride and the active cuts (the diethyl ether cuts) were chromatographed on florisil. The florisil was eluted with, successively, diethyl ether methylene chloride, ethyl acetate and acetone. The eluate was concentrated and treated with activated charcoal. The concentrate was filtered and dissolved in ethyl 25 acetate. Hexane was added to crystallize the product.

The bioactivity of the broth and subsequent recovery streams was followed by using a strain of *Byssochlamys fulva*. The components in the broth and recovery streams were visualized by chromatography on Analtech silica gel GF (Trademark) plates using neat ethyl acetate as the eluant. The developed plates were sprayed with 30 vanillin reagent (3 g of vanillin in 75 mL of ethanol and 25 mL of 85% phosphoric acid) and heated to 80°C. The product appeared as a violet spot.

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PREPARATION 3

3,4,6-Tri-O-acetyl-2-deoxy-2-trifluoro-
acetamido- α -D-glucosyl bromide

Prepared according to the method of Wolfrom et al., Journal of Organic
5 Chemistry, 32, 1821-23 (1967).

PREPARATION 4

2-Azido-3,4,6-Tri-O-acetyl-2-
deoxy- α -D-galactosyl chloride

Prepared according to the method of Lemieux et al., Canadian Journal of
10 Chemistry, 57, 1244-51 (1979).

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CLAIMS

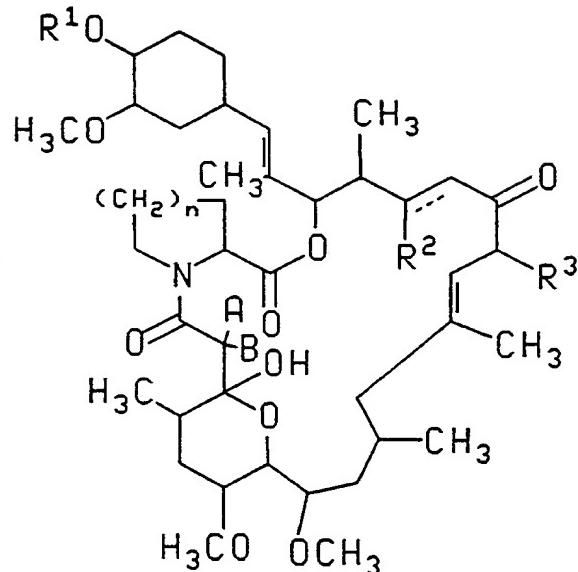
1. A compound of the formula

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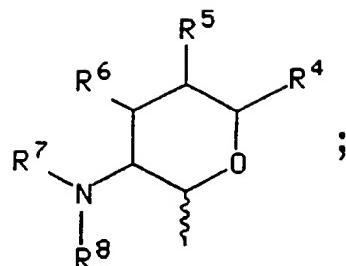


- or a pharmaceutically acceptable salt thereof;
wherein n is 1 or 2;
the dotted line represents an optional double bond in the case where R^2 is H;
25 A and B are taken separately and A is H and B is H or -OH or A and B are taken together and form =O;
 R^2 is H, (C_2C_5) alkanoyloxy or $-\text{OR}^\circ$;
 R^3 is $(\text{C}_1$ to $\text{C}_4)$ alkyl or allyl;
 R^1 and R° are each H or

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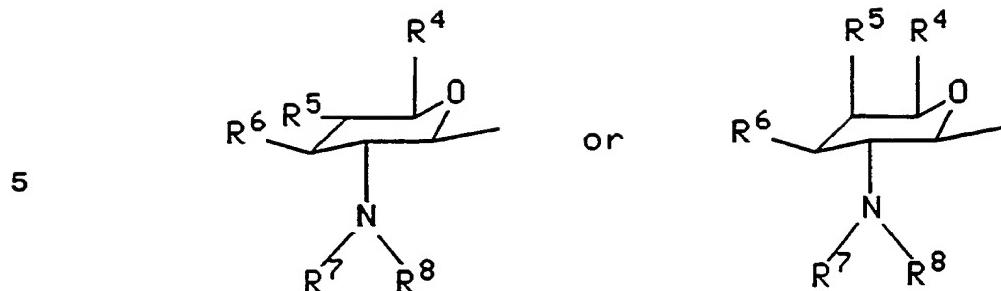


- R⁴ is, for each occurrence, independently -CO₂R⁹, -CO₂H, -CH₂OH, H, -CH₃, -CONH₂,
 10 -CONHR⁹, -CONR₂⁹, -CH₂OCOR⁹, -CH₂OCO₂R⁹, -CH₂OCONHR⁹, -CH₂OCONR₂⁹ or
 -CH₂OR⁹;
- R⁵ and R⁶ are, for each occurrence, independently (C₁ to C₄)alkoxy, benzyloxy,
 -OH, -OCOR⁹, -OCO₂R⁹ or -OSiR₃¹⁰;
- R⁷ and R⁸ are, for each occurrence, independently H, (C₁ to C₄)alkyl, -COCH₂R⁹, -COR⁹,
 15 -CO₂R⁹ or -SO₂R⁹;
- R⁹ is, for each occurrence, independently (C₁ to C₆)alkyl, (C₃ to C₆)cycloalkyl, allyl, -
 CF₃, pyridyl, thienyl, thienylmethylene, furanyl, benzyl, benzyl variously substituted with
 one to five halogen atoms, -OH groups or (C₁ to C₄)alkoxy groups, phenyl or phenyl
 variously substituted with one to five halogen atoms, -OH groups or (C₁ to C₄)alkoxy
 20 groups; and
- R¹⁰ is, for each occurrence, independently (C₁ to C₄)alkyl, phenyl or benzyl;
 provided that R¹ and R⁰ are not both H.

2. A compound according to claim 1 wherein n is 2; A and B are taken together and form =O; the dotted line represents no bond; and R² is OH.

- 25 3. A compound according to claim 2 wherein R³ is methyl, ethyl or allyl.
4. A compound according to claim 3 wherein R³ is ethyl.
5. A compound according to claim 4 wherein R¹ is

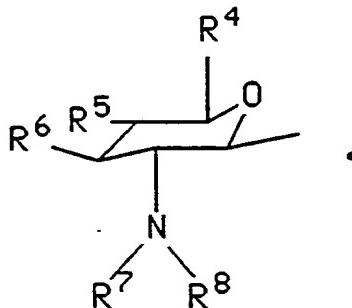
-32-



- 10
6. A compound according to claim 5 wherein R⁴ is -CH₂OH or -CH₂OCOCH₃; R⁵ and R⁶ are each independently -OH or -OCOCH₃; R⁷ is H and R⁸ is H, -COCH₃ or -COCF₃.

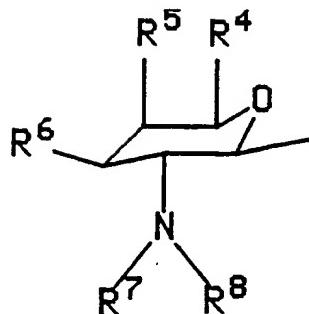
- 15
7. A compound according to claim 6 wherein R¹ is

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8. The compound according to claim 7 wherein R⁴ is -CH₂OCOCH₃, R⁵ and R⁶ are each -OCOCH₃, R⁷ is H and R⁸ is -COCH₃.
- 25
9. The compound according to claim 7 wherein R⁴ is -CH₂OCOCH₃, R⁵ and R⁶ are each -OCOCH₃, R⁷ is H and R⁸ is -COCF₃.
10. The compound according to claim 7 wherein R⁴ is -CH₂OH, R⁵ and R⁶ are each -OH, R⁷ is H and R⁸ is -COCH₃.
11. The compound according to claim 7 wherein R⁴ is
- 30 -CH₂OH, R⁵ and R⁶ are each -OH, R⁷ is H and R⁸ is -COCF₃.
12. A compound according to claim 6 wherein R¹ is

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10 13. The compound according to claim 12 wherein R⁴ is -CH₂OCOCH₃, R⁵ and R⁶ are each -OCOCH₃, R⁷ is H and R⁸ is -COCH₃.

14. The compound according to claim 12 wherein R⁴ is -CH₂OH, R⁵ and R⁶ are each -OH, R⁷ is H and R⁸ is -COCH₃.

15 15. A compound according to claim 1 wherein n is 2; A and B are each H; the dotted line represents no bond; R² is -OH and R³ is ethyl.

16. A method for treating resistance to transplantation in a mammal in need of such treatment comprising administering to said mammal a resistance to transplantation treating effective amount of a compound according to claim 1 or a pharmaceutically-acceptable salt thereof.

20 17. A method for treating autoimmune disease in a mammal in need of such treatment comprising administering to said mammal an autoimmune disease treating effective amount of a compound according to claim 1 or a pharmaceutically-acceptable salt thereof.

25 18. A method for treating fungal diseases in a mammal in need of such treatment comprising administering to said mammal a fungal disease treating effective amount of a compound according to claim 1 or a pharmaceutically-acceptable salt thereof.

30 19. A pharmaceutical composition comprising a resistance to transplantation treating effective amount of a compound of claim 1 and a pharmaceutically-acceptable carrier.

20. A pharmaceutical composition comprising an autoimmune disease treating effective amount of a compound of claim 1 and a pharmaceutically-acceptable carrier.

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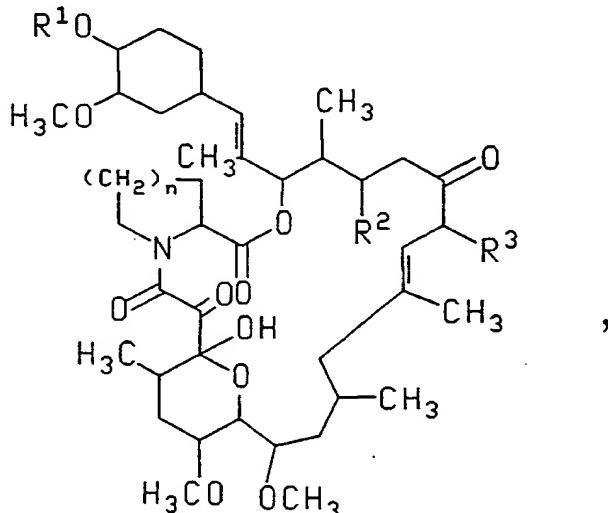
21. A pharmaceutical composition comprising a fungal disease treating effective amount of a compound of claim 1 and a pharmaceutically-acceptable carrier.

22. A process for preparing a compound of the formula

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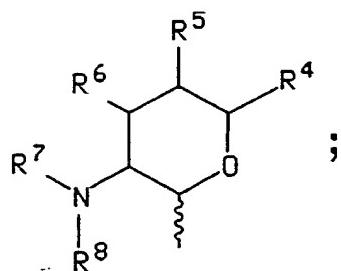
wherein n is 1 or 2;

R² is (C₂-C₅)alkanoyloxy or -OR⁰;

R³ is (C₁ to C₄)alkyl or allyl;

20 R¹ and R⁰ are each H or

25



R⁴ is, for each occurrence, independently -CO₂R⁹, -CO₂H, H, -CH₃, -CONH₂, -CONHR⁹, -CONR₂⁹, -CH₂OCOR⁹, -CH₂OCO₂R⁹, -CH₂OCONHR⁹, -CH₂OCONR₂⁹ or -CH₂OR⁹;

30 R⁵ and R⁶ are, for each occurrence, independently (C₁ to C₄)alkoxy, benzyloxy, -OCOR⁹, -OCO₂R⁹ or -OSiR₃¹⁰;

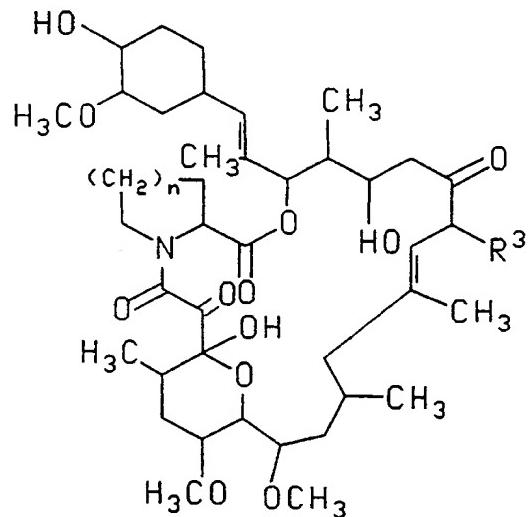
R⁷ and R⁸ are, for each occurrence, independently H, (C₁ to C₄)alkyl, -COCH₂R⁹, -COR⁹, -CO₂R⁹ or -SO₂R⁹;

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R⁹ is, for each occurrence, independently (C₁ to C₆)alkyl, (C₃ to C₆)cycloalkyl, allyl, -CF₃, pyridyl, thienyl, thiethylmethylene, furanyl, benzyl, benzyl variously substituted with one to five halogen atoms, -OH groups or (C₁ to C₄)alkoxy groups, phenyl or phenyl variously substituted with one to five halogen atoms, -OH groups or (C₁ to C₄)alkoxy groups; and

R¹⁰ is, for each occurrence, independently (C₁ to C₄)alkyl, phenyl or benzyl; provided that R¹ and R⁹ are not both H, comprising reacting a compound of the formula

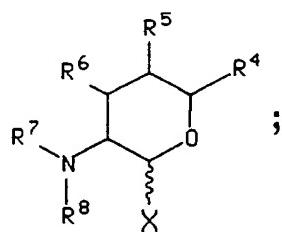
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20 wherein R³ is (C₁ - C₃)alkyl and n is 1 or 2 with 2 to 4 molar equivalents of a compound of the formula

25



wherein X is halo;

R⁴ is, for each occurrence, independently -CO₂R⁹, -CO₂H, H, -CH₃, -CONH₂, -CONHR⁹, -CON₂⁹, -CH₂OCOR⁹, -CH₂OCO₂R⁹, -CH₂OCONHR⁹, -CH₂OCONR₂⁹ or -CH₂OR⁹; R⁵ and R⁶ are, for each occurrence, independently (C₁ to C₄)alkoxy, benzyloxy, -OCOR⁹, -OCO₂R⁹ or -OSiR₃¹⁰;

R⁷ and R⁸ are, for each occurrence, independently H, (C₁ to C₄)alkyl, -COCH₂R⁹, -COR⁹, -CO₂R⁹ or -SO₂R⁹;

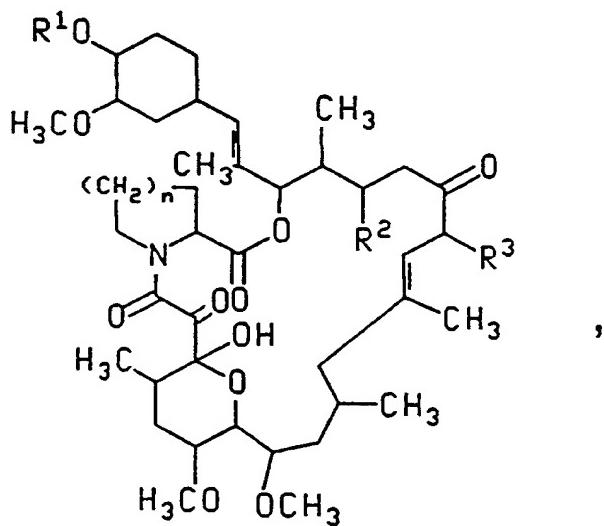
R⁹ is, for each occurrence, independently (C₁ to C₆)alkyl, (C₃ to C₆)cycloalkyl, allyl, -CF₃, pyridyl, thienyl, thienylmethylene, furanyl, benzyl, benzyl variously substituted with

- 5 one to five halogen atoms, -OH groups or (C₁ to C₄)alkoxy groups, phenyl or phenyl variously substituted with one to five halogen atoms, -OH groups or (C₁ to C₄)alkoxy groups; and

R^{10} is, for each occurrence, independently (C_1 to C_4)alkyl, phenyl or benzyl in the presence of a drying agent selected from the group consisting of molecular sieves

- 10 calcium sulfate and magnesium sulfate; a base selected from the group consisting of mercuric carbonate, silver carbonate, mercuric nitrate and silver nitrate; and a catalyst selected from the group consisting of silver triflate, silver perchlorate, silver tetrafluoroborate, mercuric trifulate, mercuric perchlorate and mercuric tetrafluoroborate in a reaction inert solvent at about -78°C to about -70°C with warming to about 0°C
15 for about 0.5 to about 24 hours and subsequent stirring at room temperature for about 0.5 to about 24 hours.

23. A process for preparing a compound of the formula



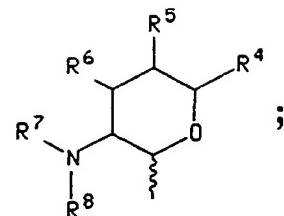
- 30 wherein n is 1 or 2;

R² is (C₂-C₅)alkanoyloxy or -OR⁰;

R³ is (C₁ to C₄)alkyl or allyl;

R¹ and R⁰ are each H or

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R^4 is, for each occurrence, independently $-\text{CO}_2\text{R}^9$, $-\text{CO}_2\text{H}$, $-\text{CH}_2\text{OH}$, H , $-\text{CH}_3$, $-\text{CONH}_2$, $-\text{CONHR}^9$, $-\text{CONR}_2^9$, $-\text{CH}_2\text{OCO}_2\text{R}^9$, $-\text{CH}_2\text{OCONHR}^9$, $-\text{CH}_2\text{OCONR}_2^9$ or $-\text{CH}_2\text{OR}^9$;

R^5 and R^6 are, for each occurrence, independently (C_1 to C_4)alkoxy, benzyloxy, $-\text{OH}$,

10 $-\text{OCO}_2\text{R}^9$ or $-\text{OSiR}_3^{10}$;

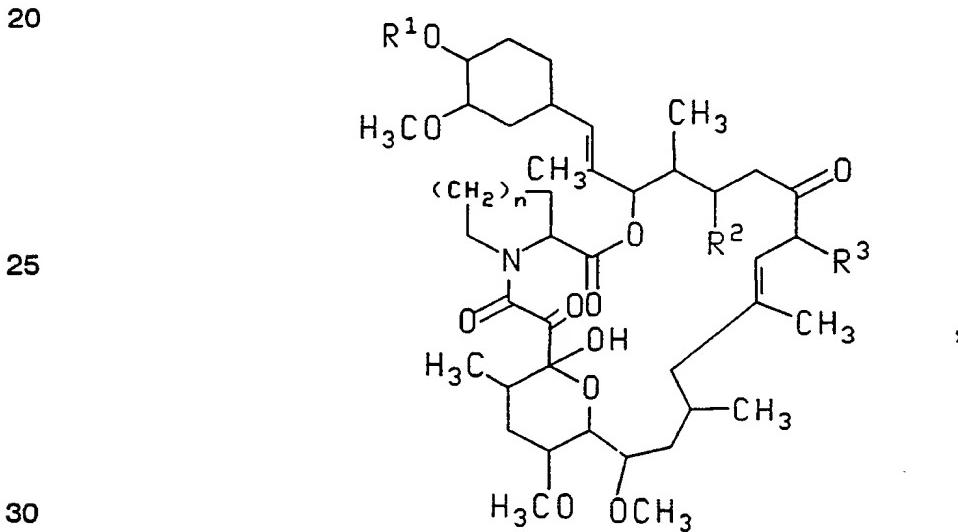
R^7 and R^8 are, for each occurrence, independently H , (C_1 to C_4)alkyl, $-\text{COCH}_2\text{R}^9$, $-\text{COR}^9$, $-\text{CO}_2\text{R}^9$ or $-\text{SO}_2\text{R}^9$;

R^9 is, for each occurrence, independently (C_1 to C_6)alkyl, (C_3 to C_6)cycloalkyl, allyl, $-\text{CF}_3$, pyridyl, thienyl, thienylmethylene, furanyl, benzyl, benzyl variously substituted with

15 one to five halogen atoms, $-\text{OH}$ groups or (C_1 to C_4)alkoxy groups, phenyl or phenyl variously substituted with one to five halogen atoms, $-\text{OH}$ groups or (C_1 to C_4)alkoxy groups; and

R^{10} is, for each occurrence, independently (C_1 to C_4)alkyl, phenyl or benzyl;

provided that R^1 and R^6 are not both H , comprising reacting a compound of the formula



wherein n is 1 or 2;

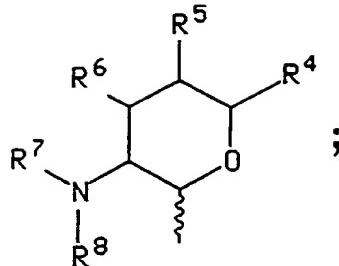
R^2 is (C_2 - C_5)alkanoyloxy or $-\text{OR}^9$;

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R³ is (C₁ to C₄)alkyl or allyl;

R¹ and R⁰ are each H or

5



- 10 R⁴ is, for each occurrence, independently -CO₂R⁹, -CO₂H, H, -CH₃, -CONH₂, -CONHR⁹, -CONR₂⁹, -CH₂OCOR⁹, -CH₂OCO₂R⁹, -CH₂OCONHR⁹, -CH₂OCONR₂⁹ or -CH₂OR⁹;
- R⁵ and R⁶ are, for each occurrence, independently (C₁ to C₄)alkoxy, benzyloxy, -OCOR⁹, -OCO₂R⁹ or -OSiR₃¹⁰;
- R⁷ and R⁸ are, for each occurrence, independently H, (C₁ to C₄)alkyl, -COCH₂R⁹, -COR⁹,
- 15 -CO₂R⁹ or -SO₂R⁹;
- R⁹ is, for each occurrence, independently (C₁ to C₆)alkyl, (C₃ to C₆)cycloalkyl, allyl, -CF₃, pyridyl, thienyl, thienylmethylene, furanyl, benzyl, benzyl variously substituted with one to five halogen atoms, -OH groups or (C₁ to C₄)alkoxy groups, phenyl or phenyl variously substituted with one to five halogen atoms, -OH groups or (C₁ to C₄)alkoxy
- 20 groups; and
- R¹⁰ is, for each occurrence, independently (C₁ to C₄)alkyl, phenyl or benzyl; provided that R¹ and R⁰ are not both H, with a catalytic amount of an alkoxide base in an alcohol solvent at 0°C.

INTERNATIONAL SEARCH REPORT

PCT/US 93/00427

International Application No.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.C1. 5 C07H19/01; A61K31/71

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
Int.C1. 5	C07H ; A61K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	EP,A,0 466 365 (MERCK & CO. INC.) 15 January 1992 see page 8, line 47 - line 52; claims 1-9 -----	1-15, 19-21
Y	EP,A,0 427 680 (SANDOZ) 15 May 1991 see page 19, line 46 - page 20, line 10 see page 21 see page 28 see page 32, line 49 - page 33, line 7; claims 1,3,7 -----	1-15, 19-21

¹⁰ Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 21 MAY 1993	Date of Mailing of this International Search Report 02.06.93
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer BRENNAN J.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/00427

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 16-18 are directed to a method of treatment of the human/animal body, the search has been carried out based on the alleged effects of the compounds.

2. Claims Nos.:

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

US 9300427
SA 69662

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 21/05/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0466365	15-01-92	CA-A-	2046040	04-01-92
		JP-A-	4230399	19-08-92

EP-A-0427680	15-05-91	AU-A-	6584390	23-05-91
		CA-A-	2029694	10-05-91
		JP-A-	3223291	02-10-91

EPO FORM P070

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

